

Regulation of CBP-Mediated Transcription by Neuronal Calcium Signaling

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Summary

The transcription factor CREB is involved in mediating many of the long-term effects of activity-dependent plasticity at glutamatergic synapses. Here, we show that activation of NMDA receptors and voltage-sensitive calcium channels leads to CREB-mediated transcription in cortical neurons via a mechanism regulated by CREB-binding protein (CBP). Recruitment of CBP to the promoter is not sufficient for transactivation, but calcium influx can induce CBP-mediated transcription via two distinct transactivation domains. CBP-mediated transcription is stimulus strength-dependent and can be induced by activation of CaM kinase II, CaM kinase IV, and protein kinase A, but not by activation of the Ras–MAP kinase pathway. These observations indicate that CBP can function as a calcium-sensitive transcriptional coactivator that may act as a regulatory switch for glutamate-induced CREB-mediated transcription.

Introduction

Studies of molecular mechanisms of learning and memory suggest that long-term changes in synaptic efficacy require changes in gene expression. Over the past decade, there has been a great deal of interest in identifying the mechanisms by which neuronal activity leads to transcriptional activation, and the cAMP response element binding protein (CREB) has emerged as the prototypical activity-regulated transcription factor (reviewed in Ghosh and Greenberg, 1995). Several lines of evidence suggest that CREB is functionally involved in mediating aspects of activity-dependent plasticity (reviewed in Frank and Greenberg, 1994). For example, activation of CREB is associated with synaptic enhancement in *Aplysia* (Dash et al., 1990; Kaang et al., 1993), with induction of long-term potentiation in the hippocampus (Bito et al., 1996; Deisseroth et al., 1996) and with light-induced shifts in circadian rhythms (Ginty et al., 1993). Additionally, mice lacking α and δ forms of CREB are defective in certain associative learning and spatial learning tasks (Bourtchuladze et al., 1994). Furthermore, in *Drosophila*, CREB is required for a learned odor avoidance task (Yin et al.,

1994, 1995) and has been implicated in regulating certain aspects of synaptic plasticity (Davis et al., 1996). These observations suggest that understanding the mechanisms by which neuronal activity regulates CREB-mediated transcription should provide important insights into the molecular basis of neuronal plasticity.

CREB was initially identified as a cAMP-responsive transcription factor that mediates transactivation via sequences that contain the cAMP response element (CRE, TGACGTCA) (Montminy and Bilezikjian, 1987; Gonzalez and Montminy, 1989; reviewed in Brindle and Montminy, 1992). It was later discovered that CREB also functions as a calcium- and growth factor-responsive transcription factor and is involved in mediating calcium-dependent transcription of a number of genes including *c-fos* and *BDNF* (Sheng et al., 1990, 1991; Dash et al., 1991; Ginty et al., 1994; Finkbeiner et al., 1997; Shieh et al., 1998; Tao et al., 1998). CREB-dependent transcription requires two transactivation domains: a glutamine-rich domain, called Q2, that functions as a constitutive transcription activator and interacts with the TBP-associated factor, hTAF130 (Ferrerri et al., 1994; Nakajima et al., 1997a), and a second domain, called the kinase-inducible domain (KID), that functions as an inducible transactivation domain. Transactivation via the kinase-inducible domain is regulated by phosphorylation of CREB at serine 133 (Ser-133) (Gonzalez and Montminy, 1989). Extracellular signals, such as glutamate and growth factors, lead to the rapid phosphorylation of CREB at Ser-133, and phosphorylation at this site is required for stimulus-dependent transactivation (Sheng et al., 1990; Ginty et al., 1993, 1994; reviewed in Ghosh and Greenberg, 1995).

The observation that CREB phosphorylation at Ser-133 is required for calcium-dependent transactivation has led to the view that CREB phosphorylation at Ser-133 might be a reasonable measure of CREB-mediated transcription. In many cases, however, CREB phosphorylation does not appear to be an accurate measure of CREB-mediated transcription. For example, although NMDA receptor activation leads to CREB phosphorylation (Ginty et al., 1993), it is not very effective in inducing CRE-mediated transcription (Bading et al., 1993). Similarly, neurotrophins lead to robust CREB phosphorylation but are not very effective in driving CREB-mediated transcription (Ginty et al., 1994; Bonni et al., 1995). It therefore seems that CREB-mediated transcription might require a second activation event in addition to CREB phosphorylation (Bonni et al., 1995). Whereas this second event could directly affect CREB, it is possible that such an event could influence molecules that couple CREB to the basal transcription machinery (Chawla et al., 1998).

Several lines of evidence suggest that CREB-binding protein (CBP) functions as a transcriptional coactivator that links CREB to the basal transcription complex (Chrivia et al., 1993). CBP was initially identified as a molecule that binds specifically to CREB phosphorylated at Ser-133 (Chrivia et al., 1993). Overexpression of CBP enhances CREB-mediated transcription (Kwok

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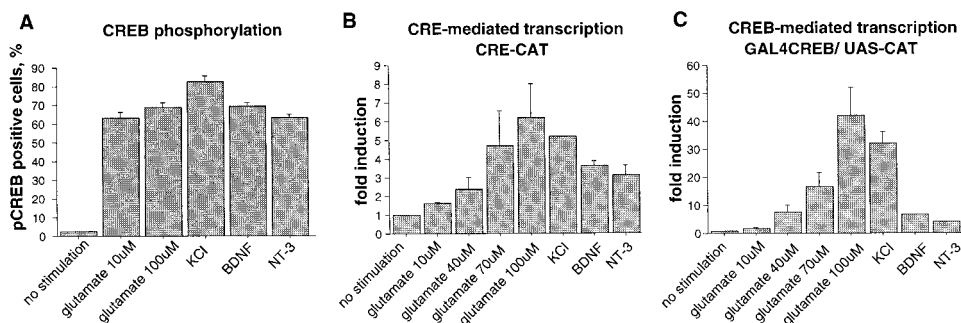


Figure 1. Glutamate Induction of *CRE*- and CREB-Mediated Transcription Requires Events in Addition to CREB Phosphorylation (A) Percentage of neurons that are phosphoCREB-positive following a 10 min stimulation of E18 cortical cultures as indicated at 5 DIV. (B) Fold induction of CAT activity in E18 cortical neurons transfected with *CRE-CAT* at 3 DIV and stimulated as indicated at 5 DIV. (C) Fold induction of CAT activity in E18 cortical neurons transfected with *GAL4-CREB* and *UAS-CAT* at 3 DIV and stimulated as indicated at 5 DIV.

et al., 1994), and microinjection of antibodies that block formation of a CREB-CBP complex inhibits CREB-mediated transcription (Arias et al., 1994). Providing CBP with a DNA-binding domain in the form of a GAL4-CBP fusion protein is sufficient for transactivation of a GAL4 upstream activating sequence (*UAS*) driven reporter in F9 and PC12 cells (Chrivia et al., 1993; Kwok et al., 1994). Based on the observations that CBP can be coprecipitated with TATA-binding protein (TBP), that it can bind to TFIIB, and that it can interact with RNA polymerase II via RNA helicase A (Kwok et al., 1994; Swope et al., 1996; Nakajima et al., 1997a, 1997b), it appears that CBP might physically link CREB to the basal transcription complex. CBP also has intrinsic histone acetyltransferase (HAT) activity that may facilitate binding of nuclear factors to chromatin by destabilizing promoter-bound nucleosomes (Bannister and Kouzarides, 1996; Ogryzko et al., 1996; Imhof et al., 1997).

While it is clear that CBP is involved in CREB-mediated transcription, the role of CBP in mediating calcium-dependent transcription is less clear. One possibility is that CBP is transcriptionally active when brought to the promoter and that CREB functions primarily to recruit this "constitutively active" transactivator to the transcription initiation complex. Alternatively, recruitment of CBP to the promoter may not be sufficient for transactivation, and calcium-dependent transcription may require a separate activation event. The possibility that CBP may be a target of intracellular signaling pathways is suggested by the fact that CBP has a number of potential phosphorylation sites and that transfection of the catalytic subunit of protein kinase A (PKA) or CaM kinase IV enhances CBP-mediated transactivation in nonneural cells (Chrivia et al., 1993; Kwok et al., 1994; Chawla et al., 1998). Here, we have explored the possibility that CBP may be a target of calcium signaling in neurons. We find that calcium influx via NMDA receptors or voltage-sensitive calcium channels (VSCC) induces CBP-mediated transactivation. CBP contains two calcium-sensitive transactivation domains that can be regulated by CaM kinases II and IV and by PKA. These observations suggest that calcium signaling to CBP is likely to be a critical event in calcium-induced CREB-mediated transcription in neurons.

Results

To determine the relationship between CREB phosphorylation and *CRE*- and CREB-mediated transcription in cortical neurons, we examined the response of the cells to a series of extracellular stimuli that should lead to CREB phosphorylation. The phosphorylation of CREB at Ser-133 following various stimuli was examined by immunocytochemistry using an antibody that specifically recognizes phosphorylated CREB (Ginty et al., 1993). As shown in Figure 1A, stimulation of cortical neurons with glutamate or KCl, or the neurotrophins BDNF or NT-3, led to comparable CREB phosphorylation in 60%–80% of the cells. The relative staining intensity under various stimulation conditions was similar (data not shown). To determine if this pattern of CREB phosphorylation was representative of *CRE*-mediated gene expression, we transfected E18 cortical neurons with a reporter construct, *CRE-CAT*, in which the chloramphenicol acetyltransferase (CAT) reporter gene was driven by the promoter of the human somatostatin gene that contains multiple *CRE* sites. Two days posttransfection, the cultures were treated with various stimuli, and CAT reporter activity was measured as an indicator of transactivation. In these experiments, the *CRE-CAT* reporter was induced by glutamatergic stimuli in a stimulus strength-dependent manner (Figure 1B). Whereas weak glutamate stimulation (10 μ M) was ineffective in driving *CRE*-mediated transcription, strong glutamate stimulation (100 μ M) led to robust transactivation of the reporter. Depolarization by KCl-induced *CRE*-mediated transcription to an extent comparable to stimulation with 100 μ M glutamate (Figure 1B). Stimulation with the neurotrophins BDNF or NT-3 also activated *CRE*-mediated transcription, but these stimuli were less effective than stimulation with glutamate or KCl (Figure 1B). These observations indicate that there is not a strict correspondence between CREB phosphorylation and *CRE*-mediated transcription and suggest that CREB phosphorylation at Ser-133 may not be sufficient to drive *CRE*-mediated transcription.

Although CREB binds to the *CRE*, *CRE*-mediated transcription is not necessarily a measure of CREB-mediated transcription since other transcription factors such

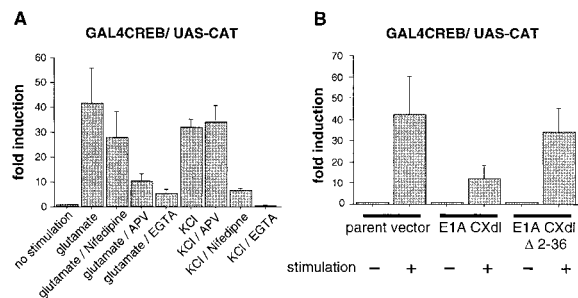


Figure 2. Glutamate Induction of CREB-Mediated Transcription Requires NMDA Receptor Activation and Is Attenuated by Inhibition of CBP

(A) Fold induction of CAT activity in E18 cortical neurons transfected with *GAL4-CREB* and *UAS-CAT* at 3 DIV and stimulated as indicated at 5 DIV (100 μ M glutamate, 200 μ M APV, 2 mM EGTA, 50 mM KCl, 20 μ M nifedipine).

(B) Fold induction of CAT activity in E18 cortical neurons transfected with *GAL4-CREB*, *UAS-CAT*, and the indicated *E1A* constructs or their parent vector at 3 DIV and stimulated at 5 DIV with 100 μ M glutamate in the presence of 10 μ M nifedipine.

as ATF-1 and CREM can also bind to the *CRE*. To examine CREB-mediated transcription directly, we transfected cortical cultures with a plasmid encoding a *GAL4-CREB* fusion protein together with a *CAT* reporter construct driven by the *GAL4* upstream activating sequence (*UAS-CAT*). As in the case of the *CRE*-driven reporter, glutamate stimulation induced CREB-mediated transcription in a stimulus strength-dependent manner (Figure 1C). *GAL4-CREB* could also mediate transactivation in response to depolarization by KCl but was only weakly activated by BDNF and NT-3 (Figure 1C). These observations indicate that CREB phosphorylation is not a reliable measure of CREB-mediated transcription and suggest that CREB-mediated transcription may require a second activation event. We should note, however, that in these experiments we are comparing CREB phosphorylation at one time point with *CRE*- and CREB-mediated transcription, and it is possible that the duration of CREB phosphorylation may be a better measure of transcription (see, for example, Bito et al., 1996).

The stimulus strength dependence of glutamate activation of *CRE*- and CREB-mediated transcription suggests that CREB-mediated transcription is a sensitive measure of glutamate receptor activation, a fact that may be important for long-term plasticity at glutamatergic synapses. Since activation of NMDA receptors is required for the induction of synaptic plasticity at glutamatergic synapses, we were interested in determining whether CREB-mediated transcription in response to glutamate stimulation also required NMDA receptor activation. As shown in Figure 2A, glutamate-induced CREB-mediated transcription was markedly attenuated by the NMDA receptor blocker APV and the calcium chelator EGTA, indicating that calcium influx via NMDA receptors is required for glutamate-induced transactivation. The VSCC blocker nifedipine only marginally attenuated the glutamate response, indicating that it contributes minimally to activation of CREB-mediated transcription by glutamate. In contrast, induction of CREB-mediated transcription in response to depolarization by KCl was inhibited by nifedipine and by EGTA,

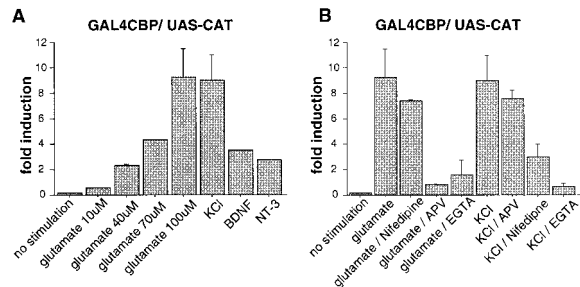


Figure 3. Glutamate and KCl Induce CBP-Mediated Transcription by a Calcium-Dependent Mechanism

(A) Fold induction of CAT activity in E18 cortical neurons transfected with *GAL4-CBP* (full length) and *UAS-CAT* at 3 DIV and stimulated as indicated at 5 DIV.

(B) Fold induction of CAT activity in E18 cortical neurons transfected with *GAL4-CBP* (full length) and *UAS-CAT* at 3 DIV and stimulated as indicated at 5 DIV (100 μ M glutamate, 200 μ M APV, 2 mM EGTA, 50 mM KCl, 20 μ M nifedipine).

but not by APV (Figure 2A), indicating that membrane depolarization can lead to CREB-mediated transcription by activation of VSCCs. These observations indicate that glutamate stimulation and membrane depolarization lead to CREB-mediated transcription by activation of NMDA receptors and VSCCs, respectively.

The ability of CREB to transactivate *CRE*-dependent genes is thought to be mediated by recruitment of CBP. To determine if CBP was required for glutamate-induced CREB-mediated transcription in cortical neurons, we used the adenovirus E1A protein to inhibit CBP. The 12S form of the E1A protein targets cellular proteins via two distinct domains. Whereas the N-terminal domain interacts and inactivates the transcriptional coactivators CBP and its homolog p300, a separate domain targets the Retinoblastoma family of tumor suppressor genes (Moran et al., 1986). A mutant of E1A, called E1A(CXdl), interacts specifically with CBP/p300 but not with Retinoblastoma family and has been used extensively to investigate the function of these proteins (Moran et al., 1986; Lundblad et al., 1995). Transfection of E1A(CXdl) in cortical cultures markedly attenuated glutamate-induced CREB-mediated transcription suggesting a requirement for CBP in this process (Figure 2B). Recent experiments have indicated that an N-terminal region of E1A (between amino acids 2 and 36) is required for E1A to bind and inhibit CBP (Arany et al., 1995). To determine if this CBP-interacting region of E1A was required for E1A(CXdl) to inhibit CREB-mediated transcription, we created a new construct, E1A(CXdl, del 2-36), which contains a deletion of amino acids (aa) 2-36. As shown in Figure 2B, transfection of E1A(CXdl, del 2-36) did not inhibit glutamate induction of CREB-mediated transcription. These observations suggest that CBP is required for glutamate-induced CREB-mediated transcription.

Since phosphorylated CREB can target CBP to the promoter, we were next interested in determining whether targeting CBP to the promoter was sufficient for transcriptional activation. We assessed CBP-mediated transcription by transfecting cortical neurons with a *GAL4-CBP* expression vector together with a *UAS-CAT* reporter. As shown in Figure 3A, full-length *GAL4-CBP*

did not activate the reporter in the absence of stimulation, indicating that recruitment of CBP to the promoter was not sufficient for transcription in neurons. Therefore, CBP appears not to function as a constitutively active transcriptional activator that simply needs to be targeted to the promoter to initiate transcription.

To determine whether CBP could function as an inducible transcription coactivator, we transfected cortical neurons with *GAL4-CBP* and *UAS-CAT* and examined if *GAL4-CBP* could mediate transcription in response to different extracellular stimuli. As shown in Figure 3A, stimulation with glutamate induced CBP-mediated transcription in a stimulus strength-dependent manner. Whereas weak glutamate stimulation (10 μ M) did not effectively induce CBP-mediated transcription, stronger stimuli led to a robust induction of CBP-mediated transcription in a stimulus strength-dependent manner (Figure 3A). Depolarization by KCl induced CBP-mediated transcription to an extent comparable to that achieved with 100 μ M glutamate (Figure 3A). The neurotrophins BDNF and NT-3 also induced CBP-mediated transcription but were less effective than stimulation with 100 μ M glutamate (Figure 3A).

To further explore the properties of stimulus-dependent CBP-mediated transcription, we examined the pharmacology of CBP-mediated transcription in response to glutamate and KCl. Glutamate-induced CBP-mediated transcription was largely inhibited by APV and EGTA but was not significantly attenuated by nifedipine, indicating that it was mediated primarily by calcium influx via NMDA receptors (Figure 3B). KCl-induced transactivation was largely inhibited by nifedipine and EGTA but was not attenuated by APV, indicating that CBP-mediated transactivation in response to membrane depolarization was mediated by calcium influx via VSCCs (Figure 3B). These results indicate that calcium influx, either through NMDA receptors or through VSCCs, can induce CBP-mediated transcription. The response of *GAL4-CBP* to various stimuli as well as to pharmacological perturbations is strikingly similar to that of *GAL4-CREB* (Figure 1C) and suggests that CREB-mediated transcription may be regulated at the level of CBP.

To identify the calcium-responsive transactivation domains of CBP, we next transfected cortical neurons with the various *GAL4-CBP* fragments and examined their ability to mediate a calcium response. In unstimulated neurons, the N-terminal fragment of CBP (aa 1–460) has a higher basal transcriptional activity than either of the other fragments (aa 721–1679, aa 1678–2441) or the full-length protein (aa 1–2441) (data not shown), suggesting that this N-terminal transactivation domain might normally be repressed in the full-length protein. Stimulation with either glutamate or KCl led to activation of both the N- and C-terminal domains but did not lead to activation of the central domain (Figure 4B). Therefore, CBP has two separate calcium-inducible transactivation domains, which can be activated by stimulation of either NMDA receptors or VSCCs.

To map the transactivation domain in further detail, we performed deletion analysis of both the N- and C-terminal domains. As shown in Figure 4C, each of the N-terminal deletion fragments (aa 1–460, 71–460, 151–

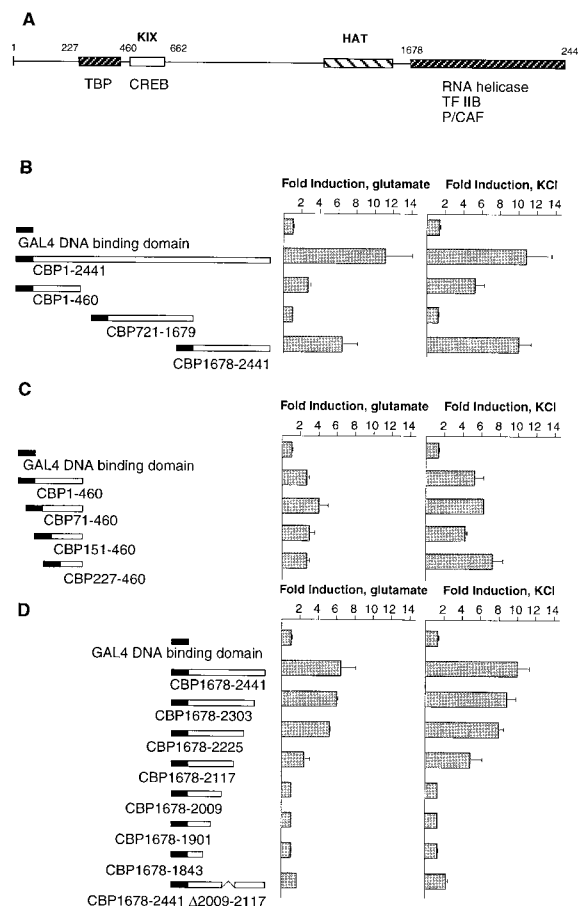


Figure 4. CBP Contains Two Separate Calcium-Inducible Transactivation Domains

(A) Diagrammatic summary of the domain structure of CBP. Regions of CBP involved in interaction with CREB, TBP, RNA helicase, TF IIB, and P/CAF are indicated (reviewed in Nordheim, 1994; Janknecht and Hunter, 1996).

(B) Fold induction of CAT activity in E18 cortical neurons transfected with various *GAL4-CBP* constructs at 3 DIV and stimulated as indicated at 5 DIV. In this and all following experiments, glutamate stimulations were carried out in the presence of 10 μ M nifedipine (to selectively activate NMDA receptors), and KCl stimulations were carried out in the presence of 100 μ M APV (to selectively activate VSCCs).

(C) Fold induction of CAT activity in E18 cortical neurons transfected at 3 DIV with constructs containing serial deletion fragments of the N-terminal of CBP and stimulated as indicated at 5 DIV.

(D) Fold induction of CAT activity in E18 cortical neurons transfected at 3 DIV with constructs containing serial deletion fragments of the C-terminal of CBP and stimulated as indicated at 5 DIV.

460, and 227–460) had similar levels of inducibility indicating that the N-terminal calcium-responsive transactivation domain was located in the region between amino acids 227 and 460. Deletion analysis of the C-terminal domain indicated that deletions up to aa 2225 did not significantly affect calcium-dependent transactivation. Further deletions between aa 2225 and aa 2009 led to a marked drop in inducibility, indicating that transactivation via the C-terminal domain required sequences between amino acids 2009 and 2225. An internal deletion construct in which aa 2009–aa 2117 were removed was

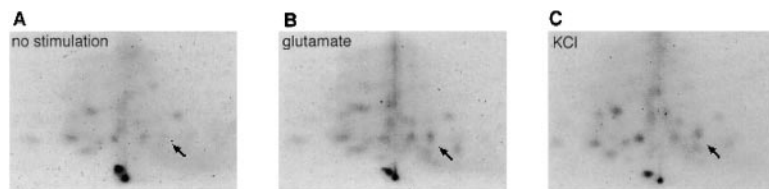


Figure 5. CBP Is Inducibly Phosphorylated by Glutamate and KCl Stimulation of Cortical Neurons

(A–C) Two-dimensional phosphopeptide maps of CBP immunoprecipitated from ^{32}P -orthophosphate-labeled cortical neurons following a 20 min stimulation with 100 μM glutamate or 50 mM KCl. The arrows indicate the location of the peptide fragment that is inducibly phosphorylated by stimulation. This is a representative example of three separate experiments in which inducible phosphorylation of CBP was apparent.

also not calcium inducible (Figure 4C), indicating that the region between amino acids 2009 and 2117 is critical for the calcium responsiveness of the C-terminal domain.

The next series of experiments were directed at understanding the mechanisms involved in calcium signaling to CBP. Since inducible phosphorylation is a common mechanism for regulating the activity of transcription factors, we examined whether CBP was a phosphoprotein and if its phosphorylation was regulated by calcium signaling. For these experiments, CBP was immunoprecipitated from ^{32}P -orthophosphate-labeled neurons, and the pattern of phosphorylation was initially examined by SDS-PAGE analysis. Analysis by SDS-PAGE indicated that CBP was a phosphoprotein but did not reveal a marked change in overall levels of phosphorylation after calcium influx (data not shown). Since change in phosphorylation at a single residue in the presence of constitutively phosphorylated residues can be difficult to detect by SDS-PAGE analysis, we carried out 2-D phosphotryptic analysis, which can reveal change in phosphorylation in individual peptide fragments. The phosphotryptic mapping of immunoprecipitated CBP revealed that several peptides in CBP are phosphorylated in unstimulated neurons, confirming that CBP is a phosphoprotein in resting cells (Figure 5A). Following stimulation with glutamate or KCl, most phosphopeptide fragments remained unchanged (Figures 5B and 5C). One peptide fragment, however, consistently showed an increased level of phosphorylation after stimulation (Figure 5, arrow; data not shown) indicating that phosphorylation of CBP can be regulated by calcium influx. By analogy with other transcription factors, it is likely that this inducible phosphorylation is involved in regulating CBP-mediated transcription.

We next examined the role of calcium-induced intracellular signaling molecules in regulating CBP-mediated transcription. Since previous studies have identified CaM kinases as being important in calcium signaling to the nucleus, we were particularly interested in exploring the possibility that they may also be involved in calcium signaling to CBP (Ghosh and Greenberg, 1995; Chawla et al., 1998; Shieh et al., 1998; Tao et al., 1998). As shown in Figure 6A, both glutamate and KCl led to the activation of CaM kinase II, albeit with somewhat different kinetics. Whereas KCl activated CaM kinase II only transiently, such that the activity peaked at 1 min and then rapidly declined to baseline, activation of CaM kinase II by glutamate was more sustained, and elevated CaM kinase II activity could still be detected at least up to 10 min

following stimulation. Analysis using an immune complex kinase assay indicated that both glutamate and KCl also led to activation of CaM kinase IV (Figure 6B). We also examined calcium activation of PKA and MAP kinase in cortical neurons since they have been shown to influence CREB-mediated transcription. KCl led to a weak activation of PKA in cortical neurons, and glutamate did not lead to detectable activation (data not shown). As previously described (Bading and Greenberg, 1991), both KCl and glutamate led to MAP kinase activation (data not shown). Therefore, the CaM kinase, PKA, and MAP kinase pathways are activated by calcium signaling in neurons and could be involved in calcium signaling to CBP.

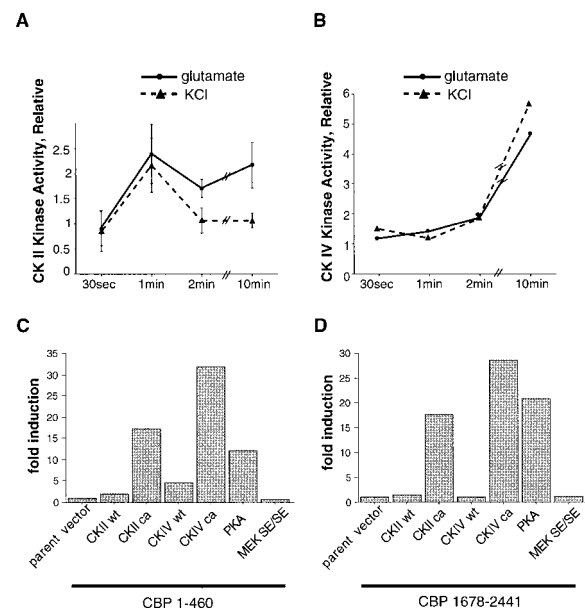


Figure 6. CaM Kinase II and CaM Kinase IV Are Activated by Calcium Influx and Can Lead to Activation of CBP

(A) Relative CaM kinase II (CKII) activity in E18 cortical neurons stimulated at 5 DIV with 100 μM glutamate or with 50 mM KCl for different durations as indicated.

(B) Relative CaM kinase IV (CKIV) activity in E18 cortical neurons stimulated at 5 DIV with 100 μM glutamate or with 50 mM KCl for different durations as indicated.

(C and D) Fold induction of CAT activity in E18 cortical neurons transfected with *GAL4-CBP(1-460)* or *GAL4-CBP(1678-2441)*, together with *UAS-CAT* and one of these constructs: wild-type (wt) or constitutively active (ca) forms of CaM kinase II (CKII) or CaM kinase IV (CKIV), catalytic subunit of PKA (PKA), or constitutively active form of MEK (MEK SE/SE).

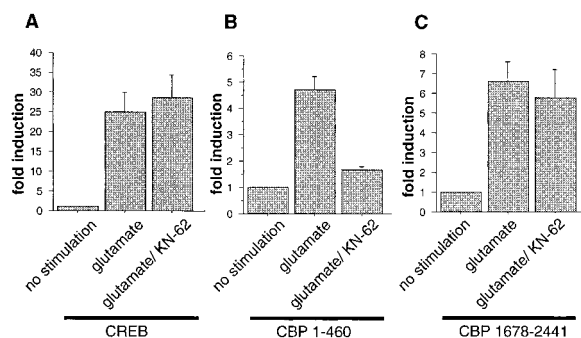


Figure 7. CaM Kinase Activity Is Differentially Required for Glutamate-Induced Activation of the N- and C-Terminal Domains of CBP (A–C) Fold induction of CAT activity in E18 cortical neurons transfected with *GAL4-CREB*, *GAL4-CBP(1–460)*, or *GAL4-CBP(1678–2441)*, together with *UAS-CAT* at 3 DIV, and stimulated as indicated at 5 DIV. Note that KN-62 inhibits glutamate induction of CBP N-terminal domain-mediated transcription, but not CREB or CBP C-terminal domain-mediated transcription.

To determine whether signaling by any of these kinases was sufficient for inducing CBP-mediated transcription, we transfected cortical neurons with constitutively active forms of these kinases together with *GAL4-CBP* fragments and *UAS-CAT*. As shown in Figures 6C and 6D, expression of constitutively active forms of either CaM kinase II or CaM kinase IV was sufficient to induce transactivation via both the N-terminal and C-terminal domains of CBP, whereas expression of the wild-type kinases had little effect. The catalytic subunit of PKA was also sufficient to induce transactivation via both the N-terminal and C-terminal domains of CBP (Figures 6C and 6D). In contrast, expression of a constitutively active form of MAP kinase kinase (MEK SE/SE) failed to induce CBP-mediated transactivation (Figures 6C and 6D), although it was effective in activating a serum response element-driven reporter (data not shown). Similarly, expression of a constitutively active form of Ras (which leads to activation of the MAP kinase pathway) also failed to induce CBP-mediated transactivation (data not shown). These observations indicate that activation of CaM kinase II, CaM kinase IV, or PKA, but not MAP kinase, can lead to the induction of CBP-mediated transcription.

To further explore the role of CaM kinase II and CaM kinase IV in calcium signaling to CREB and CBP, we examined the consequences of inhibiting CaM kinases using the pharmacological inhibitor KN-62. Inhibition of CaM kinases by KN-62 did not attenuate the ability of *GAL4-CREB* to drive expression of *UAS-CAT* in response to glutamate, indicating that CaM kinase activity was not required for glutamate activation of CREB-mediated transcription (Figure 7A). Glutamate activation of the N- and C-terminal domains of CBP was differentially sensitive to CaM kinase inhibition. Whereas glutamate induction of CBP N-terminal-mediated transcription was inhibited by KN-62, induction of CBP C-terminal-mediated transcription was unaffected (Figures 7B and 7C). Therefore, calcium signaling to the N-terminal, but not the C-terminal, domain of CBP requires CaM kinase activity. In contrast to the effects of

KN-62, pharmacological inhibitors of MEK and PKA did not attenuate calcium-induced CBP-mediated transcription (data not shown), suggesting that calcium signaling to CBP does not require activation of the MAP kinase pathway or of PKA. It is noteworthy that the distinct effects of KN-62 on CREB and CBP N-terminal-mediated transcription (Figures 7A and 7B) indicate that glutamate activation of CREB-mediated transcription does not require signaling to the N-terminal domain of CBP. Together with our experiments that indicate a requirement for CBP in CREB-mediated transcription, these results suggest that glutamate-induced CREB-mediated transcription involves both recruitment of CBP to phosphorylated CREB and activation of CBP C-terminal-mediated transcription.

Discussion

In this study, we have examined the mechanisms by which calcium influx into cortical neurons activates transcription via the CREB–CBP complex. We find that calcium-dependent activation of CREB-mediated transcription is attenuated by inhibition of CBP, suggesting that recruitment of CBP by CREB is a key event in transcriptional activation. Recruitment to the promoter, however, is insufficient for CBP to activate transcription since full-length CBP targeted to DNA by a *GAL4* DNA-binding domain cannot mediate transcription in neurons. *GAL4-CBP* can, however, mediate a strong calcium response upon stimulation of NMDA receptors or VSCCs. In a study published after this paper was submitted, Chawla et al. (1998) reported that calcium signals can activate CBP-mediated transcription in AtT20 cells by mechanisms similar to those described here. These observations suggest that calcium-induced CREB-mediated transcription requires two separate molecular events. First, CREB must be phosphorylated at Ser-133 to recruit CBP to the promoter. Second, a calcium signal must activate CBP-mediated transcription. Calcium influx through NMDA receptors or VSCCs can induce both these events and can therefore lead to CREB-mediated transcription. Our observations suggest that this dual requirement of CREB phosphorylation and CBP activation provides selectivity to the extracellular signals that can lead to CREB-mediated transcription.

The dominant view thus far has been that CBP has a constitutively active transactivation domain that is recruited by transcription factors to mediate transcription. Our findings suggest that, at least in the context of neuronal signaling, targeting CBP to the promoter is not sufficient for transcription. The fact that full-length CBP is transcriptionally silent when targeted to the promoter in cortical neurons differs from the behavior of CBP in various cell lines in which full-length CBP can mediate transactivation in the absence of stimulation (e.g., Swope et al., 1996). Since it is unlikely that CBP functions in fundamentally different ways in different cells, we suspect that the apparent constitutive activity of CBP in some cell lines may in fact be a result of signaling to CBP by mitogens and other signals that are required to maintain cell proliferation. Therefore, CBP may in general be a quiescent transcriptional coactivator that requires extracellular signals for activation.

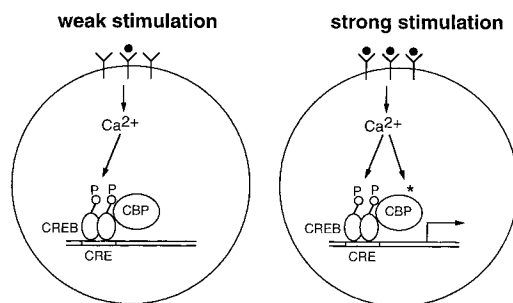


Figure 8. Diagrammatic Representation of the Proposed Mechanism by which Glutamatergic Stimuli Regulate CREB-Mediated Transcription in Neurons

Weak stimuli (low concentrations of glutamate) induce CREB phosphorylation but do not activate CBP and, therefore, do not induce CREB-mediated transcription. Strong stimuli (high concentrations of glutamate) induce CREB phosphorylation and activate CBP and, therefore, induce CREB-mediated transcription.

Our findings suggest an explanation for the observation that certain stimuli, such as low concentrations of glutamate, can lead to CREB phosphorylation at Ser-133 but not to CREB-mediated transcription. We propose that the inability of weak glutamatergic stimuli to effectively induce CREB-mediated transcription is due to their inability to effectively signal to CBP. The activation of CREB- and CBP-mediated transcription in response to strong glutamatergic stimuli is of particular interest given the observation that such stimuli are associated with long-term enhancement of synaptic strength. Our observations suggest that CBP might function as a stimulus strength-dependent switch for glutamate-induced CREB-mediated transcription and, therefore, could be critically involved in regulating long-term synaptic plasticity (Figure 8). While we observe a robust induction of *CRE*- and CREB-mediated transcription in response to NMDA receptor activation, Bading et al. (1993) have previously reported that they do not see such an activation of *CRE*-mediated transcription in hippocampal cultures. Although the source of this apparent discrepancy is not clear, one possibility is that activation of *CRE*- and CREB-mediated transcription in response to NMDA receptor activation might depend upon the cellular context.

Previous studies have identified three domains of CBP that are important for transactivation. The first is a domain that has intrinsic HAT activity (located between aa 1100 and 1600), which may facilitate binding of nuclear factors to chromatin by destabilizing promoter-bound nucleosomes (Bannister and Kouzarides, 1996; Ogryzko et al., 1996; Imhof et al., 1997). Two other domains—one in the N-terminal (between aa 227 and 460) and one in the C-terminal (between aa 1678 and 2441)—can function as transactivation domains in isolation from the full-length protein (Bisotto et al., 1996; Swope et al., 1996; Yoshida et al., 1998). Our structure-function analysis indicates that the calcium-inducible transactivation domains of CBP are nested within these N- and C-terminal domains of CBP. The N-terminal calcium-inducible domain is located between aa 227 and 460, and the C-terminal calcium-inducible domain is located between aa 2009 and

2225. A number of inferences regarding the mechanisms of transactivation can be drawn from the location of these domains within the known organization of the protein (Figure 4A). First, calcium-induced CBP-mediated transcription does not reflect recruitment of CREB to GAL4-CBP since CBP interacts with CREB via the KIX domain (located between aa 461 and 662 [Parker et al., 1996]), which lies outside of the N- and C-terminal calcium-inducible domains. Also, neither of the calcium-inducible domains of CBP includes the HAT domain of CBP, indicating that calcium activation of CBP does not require the histone acetyltransferase activity of CBP (although this domain may be important for transactivation in the context of chromatin). The domains of calcium responsiveness in CBP, however, do contain several potential protein-protein interaction sites that may contribute to calcium-dependent transactivation. The N-terminal domain includes the TBP interaction sites (Swope et al., 1996), and the C-terminal domain contains sites of potential interaction with RNA helicase A, TFIIB, and P/CAF (Kwok et al., 1994; Yang et al., 1996; Nakajima et al., 1997b). Inducible association of CBP with one or more of these molecules may contribute to calcium-dependent transactivation.

We find that CBP is phosphorylated in response to glutamate and KCl stimulation, and analogy with other transcription factors suggests that this modification is likely to be important for CBP-mediated transcription. We should note, however, that although inducible phosphorylation of CBP suggests that the activity of CBP may be regulated by calcium signaling, it is possible that CBP mediates calcium-dependent transcription by recruiting a separate calcium-regulated protein to the promoter complex. Also, since our transcription assays are based on activation of a plasmid reporter, we do not yet know if CBP-mediated transcription of endogenous genes can be regulated by calcium influx in the context of chromatin.

Our investigations of the mechanisms that mediate calcium signaling to CBP suggest that CaM kinases are likely to be involved. Both CaM kinase II and CaM kinase IV are activated by calcium influx, and constitutively active mutants of CaM kinase II and CaM kinase IV lead to CBP-mediated transactivation in the absence of extracellular stimuli. CaM kinase activity appears to be necessary for transcription via the N-terminal domain of CBP but is not required for calcium induction of CREB or CBP C-terminal-mediated transcription. While the conclusion that CaM kinase activity is not required for glutamate activation of CBP C-terminal-mediated transcription can be made with reasonable certainty (since KN-62 clearly inhibits CaM kinase II and CaM kinase IV [Enslen et al., 1994]), the conclusion that CaM kinase activity is required for activation of CBP N-terminal-mediated transcription must be made with the caveat that it is formally possible that KN-62 exerts its effects on transactivation via a mechanism other than inhibition of CaM kinases. We should also note that we have found that KN-62 completely inhibits CREB phosphorylation and CREB- and CBP-mediated transcription in response to VSCC activation (S.-C. H. and A. G., unpublished data). We are, however, reluctant to conclude that this implies a requirement for CaM kinases in VSCC activation of CREB- and CBP-mediated transcription since

there is a concern that KN-62 might directly influence VSCCs. While CaM kinases appear to be involved in calcium signaling to CBP, two pieces of evidence indicate that CBP is not a direct substrate of CaM kinase II. First, we have found that site-directed mutagenesis of the potential CaM kinase II phosphorylation sites present within the N- and C-terminal domains of CBP (at aa 77, 380, 1772, 1872, 2064, and 2355) does not significantly attenuate glutamate- or KCl-induced CBP-mediated transcription (S.-C. H. and A. G., unpublished data). Second, we have found that in an *in vitro* phosphorylation assay CaM kinase II does not phosphorylate any of the peptide fragments that are phosphorylated on CBP *in vivo* upon calcium stimulation (S.-C. H. and A. G., unpublished data).

Finally, the ability of calcium signals to induce CBP-mediated transcription suggests that CBP may be involved in calcium-dependent activation of transcription factors in addition to CREB. It is known that CBP can interact with a large number of different transcription factors such as c-Fos, c-Jun, Jun-B, c-Myb, Sap-1a, p62 (TCF), Stat1a, Stat2, SREBP, Myo-D, pp90 (rsk), GATA-1, p53, NcoA, and p160 (reviewed in Nordheim, 1994; Janknecht and Hunter, 1996). Therefore, CBP may confer calcium responsiveness upon many of these transcription factors simply by virtue of their association with CBP (see also Chawla et al., 1998). Such a mechanism is attractive since it would allow for the coordinated activation of a large group of transcription factors in response to calcium signaling, as is thought to be required for changes in synaptic efficacy that underlie long-term memory.

Experimental Procedures

Primary Cell Culture

Cortical neurons from E18 Long-Evans rat embryos were cultured as previously described (Shieh et al., 1998). The cortex was dissected in ice-cold HBSS (6.5 g/L glucose), digested in 10 U/ml papain in dissociation media (2 × 20 min), and dissociated in culture media. The dissociated neurons were plated on 60 mm plates precoated with poly-lysine and laminin at a density of 3 × 10⁶ cells per plate in glutamine-free Basal Media Eagle (Sigma) supplemented with glutamine (to 1 mM), N2 (to 1%; GIBCO), and fetal bovine serum (5%).

Plasmids

The following plasmids used in this study have been previously described: full-length, N-terminal, and C-terminal *GAL4-CBP* (Chrivia et al., 1993; Wrope et al., 1996); *E1A(CXdl)* (Moran et al., 1986); *UAS-CAT* (Martin et al., 1990); *CaM kinase II*, *CaM kinase IV*, and their constitutively active mutants (Sun et al., 1994); catalytic subunit of *PKA* (Chrivia et al., 1993); *MEK SE/SE* (Wright et al., 1997). The deletion constructs of *GAL4-CBP*: 71–460, 151–460, 1678–2303, 1678–2225, 1678–2117, 1678–2009, 1678–1901, 1678–2441Δ2009–2117, and *E1A(CXdl)Δ2–36* were generated by PCR using Pfu polymerase and were confirmed by direct sequencing.

Transfections and Stimulations

Cells were transfected by a modified calcium phosphate transfection procedure as described previously (Shieh et al., 1998). Briefly, the culture media was removed and replaced with DMEM at least 1 hr prior to transfection. The calcium phosphate/DNA precipitate was formed in HEPES buffered saline (pH 7.07) and added dropwise to the DMEM. A total of 9–15 μg of DNA was used per plate. Typically, this would include 4–5 μg of reporter construct DNA, 4–5 μg of *GAL4* fusion construct DNA, and if any, 4–5 μg of a construct encoding a candidate interacting protein (e.g., *E1A(CXdl)*). Following a 50–60 min transfection during which a fine sandy precipitate covered the cells, the cells were washed in DMEM and returned to the original

culture media. The efficiency was typically between 1% and 5%, and there was no apparent toxicity to the cells. In all cases, the DNA was purified by two rounds of CsCl banding. Cultures were typically transfected at 3 days *in vitro* (DIV) and stimulated at 5 DIV. Unless otherwise indicated, cells were stimulated at the following concentrations: 100 μM glutamate, 50 mM KCl, 100 ng/ml BDNF, and 100 ng/ml NT-3. For selective activation of NMDA receptors, cells were stimulated with 100 μM glutamate in the presence of 10 μM nifedipine; for selective activation of VSCCs, cells were stimulated with 50 mM KCl in the presence of 100 μM APV. Glutamate at 100 μM, the “strong stimulus” in these experiments, is not excitotoxic for E18 cortical neurons in culture.

CAT Assays

Cells were harvested 10 hr poststimulation in isotonic TNE (10 mM Tris [pH 7.8], 150 mM NaCl, 1 mM EDTA). Cells were spun down gently and subjected to three cycles of freeze-thaw lysis. Lysis supernatant was incubated with 0.5 μCi ¹⁴C-labeled chloramphenicol (Amersham) and 0.8 mmol acetylCoA (Boehringer Mannheim) at pH 7.8 and 37°C for 1 hr. Reaction mixtures were extracted with ethyl acetate, reduced in a speed-vac, resuspended in chloroform, spotted on thin-layer chromatography (TLC) plates (J. T. Baker), and separated by ascending chromatography for 2 hr (95% chloroform, 5% methanol). For measurements of relative CAT activity, levels of ¹⁴C emission on TLC plates were quantified by phosphorimager scans. CAT assays and histograms shown are representative examples of experiments, which were repeated two or more times. Any single histogram represents results of experiments performed on cells that were simultaneously cultured, transfected, and assayed to minimize variability due to subtle differences in Experimental Procedures.

Kinase Assays

The kinase assay for CaM kinase II was performed using the calcium/calmodulin-dependent protein kinase assay system (GIBCO). Briefly, cell extract was prepared by dounce homogenization, and volumes of lysis supernatant containing equal amounts of protein, as determined by the Bradford assay, were used for the assay. The lysate was incubated at 30°C for 3 min with 5 × 10⁵ dpm [^γ-³²P] ATP and 15 μM autocalmitide 3, which is a CaM kinase II-specific substrate peptide, in the presence of 1 mM CaCl₂ and 20 μg/ml calmodulin (for total activity) or 1 mM EGTA (for calcium-independent activity). The reaction was stopped by adding ice-cold trichloroacetic acid. Following a brief centrifugation, the supernatant was spotted onto the P81 phosphocellulose paper and incorporated ³²P was measured using a scintillation counter. The ratio of calcium-independent activity to total activity was used as an index of activated CaM kinase II. The immune complex kinase assay for CaM kinase IV was performed using a previously published method (Park and Soderling, 1995). Briefly, 5 μg of anti-CaM kinase IV antibody (Signal Transduction) and 40 μl of a 50% slurry of a 1:1 mixture of protein G Sepharose and protein A Sepharose (Pharmacia) were added to volumes of lysis supernatant containing equal amounts of protein. CaM kinase IV activity was measured by ³²P incorporation into syntide-2 in the presence of 0.8 mM CaCl₂ and 1 μM calmodulin (for total activity) or 1 mM EGTA (for calcium-independent activity).

Two-Dimensional Phosphopeptide Mappings of CBP

For analysis of phosphopeptides after *in vivo* labeling, E18 cortical neurons in 60 mm dishes were labeled for 4 hr at 37°C in the presence of 1 mCi/ml of ³²P-orthophosphate (ICN) in phosphate-free media. Following indicated stimulation, the cells were rinsed with cold PBS, harvested, and lysed for 20 min at 4°C in a buffer containing 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 100 μg/ml PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM sodium orthovanadate. After centrifugation at 13,000 rpm for 10 min, the supernatant was incubated with 2 μg anti-CBP antibody (Santa Cruz) for 2 hr at 4°C. The immune complex was isolated by addition of 60 μl of a 50% slurry of protein A Sepharose (Pharmacia) for 1 hr, washed three times with the lysis buffer and once with PBS, then eluted by boiling SDS lysis and resolved by the 6% SDS-PAGE. The radioactive band corresponding to CBP

was visualized by autoradiography, excised from the gel, and subjected to two-dimensional phosphopeptide mapping analysis as described before (Boyle et al., 1991). For analysis of phosphopeptides after *in vitro* phosphorylation by CaM kinase II, CBP was immunoprecipitated and was incubated for 30 min at 30°C in a reaction mixture of 50 µl containing 50 mM HEPES (pH 7.5), 10 mM MgCl₂, 1 mM CaCl₂, 1 µM calmodulin, 200 µM[γ-³²P] ATP (5000 cpm/pmol), and 20 ng/µl purified CaM kinase II. Two-dimensional phosphopeptide analysis was then performed as described above.

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